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(54) Title: ANTI-PARATOPIC ANTIBODY AS AN IMMUNOGEN

(57) Abstract

The present invention provides a method of manufacture of an anti-paratopic antibody comprising the steps of: (1) selecting from a pool of antibodies occurring in one species a prototypic set the members of which are effective in binding a specific antigen (or antigen epitope), and (2) utilizing one or more members of said prototypic set, or paratopic fragments thereof, as an immunogen in a host of a different species, or in an in vitro incubation system comprising cells derived from the same or a different species, to produce antibodies having a characteristic which is anti-paratopic with respect to said immunogen to produce a synthetic replicate of the specific antigen or epitope. Antigen (or antigen epitope), and monoclonal antibodies, vaccines and processes of immunisation employing the product of the method of manufacture are also described.

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"ANTI-PARATOPIC ANTIBODY AS AN IMMUNOGEN"

Field of the Invention

This invention relates to immunology and more particularly to a method of manufacture of immunogenic, compositions, to immunogens manufactured by the method, and to antibodies manufactured therefrom.

Background Art

An immunogen is a molecule capable of eliciting an immune response in a vertebrate. The response elicited is believed to be determined by topographical shape characteristics of the immunogen. Immunogens are also called antigens i.e. ANTIbody GENerators because one aspect of the induced response involves the production of antibody molecules whose function is to lock onto the immunogen. Those areas of the immunogen to which the antibody molecule binds are variously referred to as the antigenic determinants, epitopes or haptens. The last term, namely hapten, is generally associated with the term carrier and this term refers to that part of the

immunogen/antigen which interacts with cellular components of the vertebrate immune system.

These regions on the immunogen and the names used to define them should not be regarded as absolute. Thus the genus of vertebrates has immune systems which will recognize immunogens; but not all species necessarily recognize the same molecular areas as being haptenic areas or carrier areas. Within a species this can only be determined experimentally. Thus mice will not necessarily process immunogens in the same way as would, for example, the immune system of Man. Furthermore, within a species, individual specimens will not respond to the same degree. This is because the immune response to an immunogen has a genetic (hereditary) component. Thus some individuals will respond better to an immunogen while others may not respond at all.

The immune response to an immunogen is an integrated phenomenon in that a class of white blood cells called T lymphocytes, for example, reacts with the carrier determinants which in turn allows a class of white blood cells called B lymphocytes to transform and start producing antibodies to the antigenic determinants.

Each cell recognizes only one determinant and each antibody producing B cell (plasma cell) generates only antibody molecules of one given specificity. Hence the immune system is said to be highly specific. Upon stimulation these plasma cells multiply and thereby give

rise to a clone of identical antibody secreting cells. If it were possible to isolate these identical antibody secreting cells, they would be referred to as monoclonal and the antibodies referred to as monoclonal antibodies.

Figure 1 is a diagrammatic illustration of the response of a mouse to an immunogen/antigen. Under normal conditions each monoclonal antibody generated by the mouse in vivo mixes with other monoclonal antibodies so that a polyclonal antibody response eventuates.

Each antibody comprises a glycoprotein molecule. The portion of an antibody molecule embodying the characteristic of shape or molecular topography, or code sequence which enables it to bind and so for example neutralise the antigenic determinant or epitope of an antigen is known as a "paratope". The paratope is conceptually a molecular region of a shape complimentary to the epitope or to a part of the epitope of the antigen and is thought to reside in the so called hypervariable region of the antibody glycoprotein molecule.

Antibody producing lymphocytes are present in high concentration in the spleen but antigen reactive spleen lymphocytes cannot readily be cultured in isolation. However mono-clonal antibodies may be manufactured and isolated therefrom by use, for example, of techniques of hybridoma technology. In one such technique mice are first exposed to an antigen whereby the mouse develops antibodies. With reference to Fig. 2, spleen cells of

the immunised mouse are fused with mouse myeloma cells. The growth of hybrid cells is promoted and the hybrids are screened for specific antibody secretion. Those useful are cultured or undergo further genetic stabilisation procedures. By this means specific mono-clonal antibodies may be produced and isolated.

Selected antibodies, or mixtures thereof such as are produced in the method of Fig. 2, may be used to neutralise an antigen in an organism, a paratope of each antibody in effect forming a complex with an epitope of the antigen.

In anti-idiotypic immunology a second stage process shown in Fig. 3 is involved. Mouse 1 is first immunised with an antigen. Thereby giving rise to several clones of antibody producing cells. One cell line is chosen on the basis of the characteristics of the generated antibody and the antibody is referred to as Ab1. Ab1 is then used to immunise a second mouse - mouse 2. The latter must have a genetic constitution very similar to, or identical with that of mouse 1. Mouse 2 generates monoclonal antibodies to Ab1, a subset of which may be directed against the paratope of Ab1. All the antibody subsets generated by mouse 2 against Ab1 may be referred to as Ab2 though the Ab2 subset specific for the paratope of subset 1 is sometimes referred to as Ab2 beta. The second mouse mono-clonal antibody, Ab2, has an anti-paratope, that is to say having a molecular portion

with a shape characteristic complementary to the paratope of the first antibody. If the epitope of the original antigen is considered to be "mould positive", then the paratope of the mono-clonal antibody Ab1 can be considered to be a counterpart or "mould negative" and the paratope of the anti-Ab1 antibody that is the paratope of the Ab2 mono-clonal antibody can be considered to replicate the "mould positive". It will be understood however that in each case the replication is not exact. When used in a vaccine, the second mono-clonal antibody, Ab2, functions as a harmless immunogen which stimulates production of Ab3 antibodies in the vaccinated animal effectively producing immunity to the first antigen.

Disclosure of the Invention

According to one aspect the present invention consists of a method of manufacture of an anti-paratopic antibody comprising the steps of:

(1) selecting from a pool of antibodies occurring in one species a prototypic set the members of which are effective in binding a specific antigen (or antigen epitope), and

(2) utilizing one or more members of said prototypic set, or paratopic fragments thereof, as an immunogen in a host of a different species, or in an in vitro incubation system comprising cells derived from the same or a

different species, to produce antibodies having a characteristic which is anti-paratopic with respect to said immunogen to produce a synthetic replicate of the specific antigen or epitope.

In a preferred embodiment of the invention the anti-paratopic mono-clonal antibodies are then used to immunise a member of the same species as that from which the prototypic set was selected.

For preference the pool of antibodies consists of naturally occurring human antibodies.

The prototypic set is a set of antibodies selected on the basis of effectiveness against a particular antigen, or epitope thereof, for example is a set of human antibodies obtained from humans carrying antibodies resulting from exposure to HIV.

The antibodies, or prototypic paratope bearing segments of them, are utilized as an immunogen in a mouse host to produce mouse antibodies having anti-paratope characteristics.

The mouse antibodies are then screened for effectiveness for inducing, in humans, antibodies which bind the HIV.

In a second embodiment of the invention the anti-paratopic monoclonal antibodies are then used to immunise a member of a third species differing from that from which the prototypic set was selected or from which

the anti-paratopic monoclonal antibodies were derived.

Brief Description of the Drawings

Figure 1 is a diagrammatic illustration of the response of a mouse to an immunogen/antigen.

Figure 2 is a diagrammatic representation of monoclonal antibody production.

Figure 3 illustrates anti-idiotypic antibody Ab2 production.

Figure 4 is a schematic representation of the method of manufacture of anti-paratopic antibodies according to the invention.

Figure 5 (I) illustrates a general procedure for the purification of HIV positive human antibodies..

Figure 5 (II) illustrates a general procedure for the purification of HIV antigen specific human antibodies.

Figure 6 (a) illustrates purifcation of human IgG prior to delineation into HIV/HIV antigen specific antibodies.

Figure 6 (b) illustrates purification of human IgA prior to delineation into HIV/HIV antigen specific antibodies.

Figure 6 (c) illustrates purification of human IgM prior to delineation into HIV/HIV antigen specific antigens.

Preferred embodiments of the invention have a number of advantages over the prior art.

Firstly, the invention produces a mouse

anti-paratope which is a counterpart of a naturally occurring human antibody paratope for a specified antigen. Upon inoculation the mouse anti-paratope mono-clonal antibody produces in a human an antibody bearing a replica of the naturally occurring human paratope.

In the prior art there was produced a mouse anti-paratope which was a counterpart of an artificially generated mouse antibody. Such a mouse anti-paratope mono-clonal antibody would produce in a human an antibody bearing a replica of an artificially created mouse paratope
(in contrast to a human paratope) and which may not be as effective in binding the specific antigen in a human. In relation to the prior art the invention does not rely upon the assumption inherent within the prior art that the mouse processes antigen in exactly the same way as humans.

Secondly, in comparison with the prior art scheme illustrated in Fig. 3, the invention provides a more direct general route shown schematically in Fig. 4 to the production of an anti-idiotypic antibody.

Thirdly, since preferred embodiments of the invention use widely available and naturally occurring, i.e. endogenous, antibodies as the starting material rather than antigens, the process is expected to be less costly to conduct.

Fourthly, the process is safer to conduct than processes requiring handling for example of potentially harmful virus antigens.

Best Modes of performing the Invention

An embodiment of the invention will now be described by way of example only. The embodiment concerns the manufacture of a vaccine to confer immunity against Acquired Immune Deficiency Syndrome (AIDS). The invention is not however limited to use for production of any particular vaccine, and has uses other than for the production of vaccines.

The manufacture may be considered as involving the steps of:

(1) selecting a prototypic set of antibodies;

(2) preparing one or more immunogens therefrom;

(3) inoculating hosts with the one or more immunogens;

(4) generating a monoclonal antibody pool from each host;

(5) screening the monoclonal antibody pools;

(6) testing the screened antibodies for effectiveness as a vaccine.

In the example under consideration the first stage is to select from the pool of human antibodies a prototypic set, in this case a set of immunoglobulins which effectively bind the aetiologic agent for Acquired Immune Difficiency Syndrome (AIDS). The generally

accepted aetiological agent for AIDS is currently known as Human Immunodeficiency Virus hereinafter referred to as HIV.

That is accomplished by obtaining human immunoglobulins from individuals exposed to HIV. About 75% of such individuals have antibodies to HIV.

The antibodies from these individuals are screened for effectiveness in binding HIV antigens and/or antigenic fragments. Those antibodies effective at this function are retained as members of the prototypic set i.e. they are a subset of the pool of human immunoglobulins.

If desired the retained immunoglobin members so selected may be purified and used directly in step (3).

Preferably, however, in a second step the human immunoglobulins ("Ig") are subdivided into classes G, A, M, D, E (to use the WHO designation) and more particularly identified in Table I.

Table 1

PHYSICAL PROPERTIES (F MAJOR HUMAN IMMUNOGLOBULIN CLASS IN SERUM

WHO Designation	IgG	IgA	IgM	IgD	IgE
Sedimentation Coefficient	75	75,9S,11S*	19S#	75	85
Molecular Weight	150,000	160,000+ dimer	900,000	185,000	200,000
Number of Ig Units	1	1-2	5	1	1
Number of Antigen Binding Sites	2	2-4	10	2	2
Identity of Heavy Chain	(gamma)	(alpha)	(mu)	(delta)	(epsilom)
Carbohydrates Content	3	8	12	13	12
% Total Immunoglobulin in normal human serum	80	13	6	0-1	.002
Concentration range in normal human serum	8-16 mg/ml	1.4-4 mg/ml	0.5-2 mg/ml	0-0.4 mg/ml	17-450 mg/ml

* IgA dimer found in mucosal (secretory) immune system. It is complexed with a secretory component (MW=60,000) and J chain (MW=15,000).

\# IgM contains J chain.

Source: I.M. Roitt, Essential Immunolgoy, 4th Ed. Blackwell 1980

More desirably still the immunoglobulins are further divided into sub-classes, for example, IgG being divided into four sub-classes, IgA being divided into two sub-classes and IgM into two sub-classes. In the preferred embodiment each of sub-classes IgG 1-4, IgA 1-2 and IgM 1-2 are purified and isolated from each other. IgD and IgE sub-classes are present in immunoglobin in small concentration and their inclusion is optional.

The human IgG/A/M is drawn from the three main groups affected by the AIDS viral infection, viz

- male homosexuals
- bisexual/female/heterosexual AIDS carriers.
- haemophaelics

The blood plasma is heated to 56°C to kill the virus. Cellular components and serum debris are removed either by aspiration of the serum component or by centrifugation (in the case of plasma).

Human IgG can be purified free of all non-IgG contaminants by affinity chromatography. Other procedures such as ion-exchange chromatography may be used but affinity chromatography is preferred for speed and selectivity. More particularly purification is generally effected by means of chromatography using PROTEIN-A SEPHAROSE beads (obtainable from e.g. Pharmacia Biotechnology Pty. Ltd.)

Subclasses of IgG may also be isolated by chromatography.

In a similar manner human IgA purification may be carried out by anti-IgA affinity chromatography.

Human IgM may be purified by a combination of

(a) Protamine sulphate chromatography, and

(b) Column chromatography, or

(c) IgM affinity chromatography.

The purified prototypic immunoglobulins set may be used directly as an immunogen for inoculation of mice in stage 3.

Alternatively the Ig subclasses may be screened to select antigen specific antibodies for use as the immunogen. In this case, the Ig sub-classes are next screened for effectiveness against HIV antigen to select the most effective sub-classes in binding the antigen. More preferably the antigen is first divided into sub-classes known as p18, p24, gp41, p55, gp120 and gp160. These antigen sub-classes differ from each other in molecular structure and can be separated by SDS-polyacrylamide gel electrophoresus. Each Ig subclass is then screened against each antigen subclass to select the most effective Ig's.

TABLE 2

HTLV III - HUMAN SERUM Ig PARATOPE GRID

Human Serum Ig's* / HTLV III Antigens	No. of Epitopes	IgG (80%)				IgA (13%)		IgM (6%)		Antigen Specific Human Paratope
		1 (65%)	2 (23%)	3 (8%)	4 (4%)	1 (80%)	2 (20%)	1	2	
p24	2									16
gp41	4									32
p53	5									40
gp120	12									96
gp160	16									128
Carrier Specific Paratope		39	39	39	39	39	39	39	39	
Total Number per Ig Class		166				78		78		
% Spread of Paratope		50%				25%		25%		

* IgD and IgE not included

With reference to Table 2 there is shown a "paratope grid". If it is assumed that there is one antigenic group anchored to a ten thousand dalton carrier group then the total number of antigenic groups (epitopes) available among the five antigen subclasses would be thirty nine. With eight potential antibody classes in the grid that can respond to the thirty nine antigens the total number of possible antibodies carrying paratopes specific for HIV is 312. Put differently there are on average thirty nine HIV paratopic bearing human immunoglobulins per immunoglobin sub-class. Thus, for example, it might eventuate that human IgG1 has specificities for all thirty nine epitopes ("haptens"), i.e., there would be thirty nine IgG1 molecules all absolutely identical except for one feature namely their Fab paratope would be different.

In the third step of the embodiment, one or more members of the prototypic set are used as an immunogen in a non-human host for example by being injected into a mouse. The one or more members are preferably the most effective of the immunoglobin sub-classes. The criteria of effectiveness may be effectiveness against a specific antigen or effectiveness against a spectrum of antigen sub-classes or other criteria.

Human antibodies are excellent immunogens when injected into mice. The antigenic sites on the human antibody molecules are spread right across the length of

the molecule from the NH2 terminii-ie the Fab end to the carboxylic acid terminus -ie the Fc end. The Fab NH2 end carries the paratope. Other antigenic components of the Fab are present for structural or "carrier" purposes. For the purposes of the vaccine the Fc exclusively exhibits "carrier" as opposed to paratope antigens.

Immunization studies have demonstrated that not all the antigenic sites on the intact human immunoglobin molecule are of equal value in that a greater proportion of induced antibodies tend to be directed against the Fc region. This phenomena is described as antigenic competition or more accurately as intramolecular antigenic competition. When developing an antiparatopic antibody however the part of the molecule of most interest is the Fab area that is to say the paratope bearing region. A simple way to overcome the problem of Fc dominance is to enzymatically cleave the immunoglobin molecule and isolate the Fab fragment. When used to immunize a mouse this will cause all the induced immunoglobulins to be directed against the Fab fragment. A subset of the anti Fab antibodies generated by the mouse, irrespective of whether an intact immunoglobin molecule or a Fab/F(ab)'2 fragment has been used, will be directed against the internal idiotope i.e. paratopic image of the human immunogen. Thus the member of the anti HIV prototypic set used as an immunogen in the mouse may be either (a) the mixed intact human immunoglobin

specific for the AIDS virus, (b) selected classes or subclasses of the intact immunoglobin, (c) a Fab/F(ab)'2 fragment of one or a combination of the AIDS specific immunoglobulins or (d) a Fab/F(ab)'2 fragment of one or a combination of the AIDS specific immunoglobulins complexed to carries eg. Keyhole Limpet Haemocyanin or human albumin.

The stage of preparation of immunogen may thus include enzymatic digestion or chemical cleavage of the human anti HIV immunoglobin and conjugation of the Fab/F(ab)'2 to microspheres or the like.

As will be apparent from the foregoing, it is conceivable that when injected into the mouse, the HIV IgG1 subgroup could provide all the relevant paratopes on one type of carrier. This regime would favour the generation of anti-idiotypes in the mouse (as opposed to the generation of anti-"carrier" molecules).

There is a possibility though of inter-molecular antigenic competition so that only a small variety of the human paratopes directed against HIV will end up being antigenic in the mouse. If this occurs then there are various ways of proceeding:

(i) After the screening step those paratopes that are dominant could be isolated from the immunogen population and a second immunization carried out to develop mouse anti-paratope antibodies to the remaining paratopes.

(ii) The anti-idiotypes/idiotypic reagents arising from

the first immunization could be screened and tested to see if the anti-idiotypes/idiotypic reagents cover the known HIV antigens/antigenic fragments. If all the known antigens are covered by the generation of anti-idiotypes/idiotypic reagents then a second immunization protocol may not be necessary.

(iii) A different mouse strain could be employed. The eventual manufacturing route thus depends on whether anti-idiotypes/idiotypic reagents to all reagents are required.

It may suffice to have, say, one or two of the haptens from each antigen group covered. To a great extent though, this is an issue that will be resolved by the mouse itself in that it may only be able to raise anti-idiotypes/idiotypic reagents against a restricted idiotype range.

How all these factors are weighted will determine the nature of the immunogen that is preferred for injection into the mouse.

Thus it may be preferable

(i) to choose a particular class/subclass of HIV+ve human immunoglobulin which expresses several specificities and use this to immunize the mouse.

Alternatively

(ii) given the diversity of the immunoglobulin response the antibody range may not be restricted and a more general immunization routine adopted. In the latter case

(iii) subclass purification may be called for coupled to several primary immunizations.

An excellent starting position though would be to opt for (i) and then remove the Fc prior to further development of the immunogen by linking it to adjuvants such as precipitated immunoglobulins or microspheres.

While stating a preference for (i) an outline of the various alternative pathways for the purification and preparation of the immunogen is shown in Figs. 5 and 6.

After injection of the immunogen into mice, preferably after a second immunization, mouse spleen cells are harvested by normal methods and fused to NSI in accordance with conventional hybridoma technology. Hybrids are then grown and screened and positive hybrids cloned and re-tested. The clones are then adapted and grown in serum-free media and specific antibodies purified and ready for testing in humans.

The monoclonal antibody pool may be generated using for example the standard method or the "LOTTO" method as outlined in Table 3 below:

TABLE 3

STANDARD METHOD		"LOTTO"	
one-hit		(multi-chance)	
	(i) immunize		
(ii)	4 wks later boost	(ii)	2-3 days later boost
(iii)	4 days later	(ii)	2-3 days later
	spleen cell prepn		cell prepn
(iv)	Hybridoma productn	(iii)	Hybridize
(v)	Preliminary screen	(iv)	Preliminary screen
(vi)	CLONE	(v)	Clone

The Fab pool may be screened by conventional means as shown in Table 4:

TABLE 4

SCREENING FOR FAB POOL

Antigen	Mc Ab Configuration (+Ve/-Ve)					
	1	2	3	4	5	6
Bence-Jones	+	-	+	-	+	-
Human Ig	+	+	-	+	-	-
Immunogen	-	-	+	+	-	+
ACTION						
Discard/Retain	D	D	D	D	D	R

The anti-idiotype pool may be screened by conventional means. For example HIV on a tray is mixed with human anti-HIV antibodies before and after incubation with mouse HIV idiotype complexed to microsphere/eupergit spheres, then chased with anti-mouse Ig-PO, +Ve is discarded, and -Ve is retained.

Alternatively HIV on beads is mixed with human anti-HIV PO-enzyme + mouse HIV idiotype +Ve response is discarded.

As will be appreciated by those skilled in the art the antibodies may be selected from a pool occurring in a different species of veterbrate and the prototypic set may be selected for effectiveness against a different antigen. The antibodies may not be free in plasma but may be bound to cells (e.g. B cells) or may exist as immune complex. The prototypic set may be divided into members using different criteria from that exemplified.

Other methods may be used for separation such as use of dyes bound to inert supports, or the use of monoclonal antibodies, etc. and purification of the immunogen without departure herefrom.

The immunogens, or fragments thereof may be utilized in host species other than mice.

The antibodies so obtained may be used in various ways for example for immunization of the verterbrate from which the antibodies were obtained, in test methods and for other purposes.

The invention will now be described more specifically by way of the following Example.

PREPARATION OF HUMAN IMMUNODEFICIENCY VIRUS SPECIFIC HUMAN ANTIBODIES

A. PREPARATION AND PURIFICATION OF HIV ANTIGENS.

Native and recombinant antigens can be purified by affinity chromatography using human antibodies or antibodies from another species such as mouse monoclonal antibodies specific for the HIV antigens. By way of illustration the procedure described will be that using human antibodies. There is very little difference between the two approaches though the benefit is that with the appropriate mouse monoclonal antibodies specific antigens can be purified if the antigen source is the native one. If the antigen source is a recombinant one then human antibodies will allow for the specific purification of the recombinant antigen. When human antibodies are used the steps involved are

(1) the preparation of human IgG from HIV infected individuals

(2) the preparation of the human antibody (IgG) column and

(3) the purification of the viral antigens using the aforementioned column.

1. Preparation of a human antibodies.

According to this procedure human antibodies were first purified by either hydroxyapatite chromatography,

ion-exchange chromatography (DEAE-cellulose) or protein-A affinity chromatography. By way of example the method for the purification described is that of protein-A agarose column chromatography.

Pooled human sera was obtained from patients positive for the AIDS virus as determined by both an AIDS antibody ELISA assay and subsequently confirmed by the Western Blot assay. Prior to use the serum had been heat treated (56°C 30 mins). A 2.0ml protein-A agarose column was washed with 20ml of the Monopure bindng buffer (Pierce). 4mls of the pooled serum was diluted with 8mls binding buffer and centrifuged (2000xg:10 min:RT). The supernatant was applied to the column, allowed to percolate through and exhaustively washed in the binding buffer. The human IgG was specifically eluted using the commercially obtained elution buffer (Pierce). Following dialysis and concentration, the A_{280} data was used to determine the concentration of protein which was calculated to be 30 milligrams as determines by the $E^{1\%}$=1.43(280nm). Western Blot and ELISA data confirmed the presence of HIV specific antibodies in the IgG fraction purified in this manner.

2. The preparation of the IgG affinity column.

30 mgs of the human IgG was equilibrated in the coupling buffer (0.1M $NaHCO_3$ pH8.3 + 0.5M NaCl) and mixed with 4 gms CnBr-Sepharose4B (Pharmacia) which had been pre-washed in 1mM HCl, swollen and equilibrated in

the coupling buffer. The mixture was mixed end-over-end in a sealed coupling vessel (2hrs, RT). Unreactive groups on the matrix were blocked using 0.2M glycine in the coupking buffer (16 hrs, 4°C) and the ensuing IgG-Sepharose matrix exhaustively washed in high salt and variable pH buffers prior to the purification of the HIV antigens.

3. The purification of the native/recombinant antigens.

By way of illustration the method described is that for the recombinant HIV antigens in particular recombinant 'gp120'.

Sub genomic clones of HIV cDNA encoding gp120, gp41, p24, and p18 were cloned and amplified in E. Coli using λ gt11. The E. Coli lysates were screened with in-house and by commercial HIV antigen ELISA's. Radioimmunoprecipitation studies confirmed the presence of recombinant HIV antigens and the molecular weights of the recombinant antigens were as predicted e.g. 60kD for the recombinant 'gp120'.

Following precipitation of E. Coli antigens with $(NH_4)_2SO_4$ the supernate was concentrated (Amicon)dialysed against distilled water and then against 0.05M Phosphate buffer pH7.2(16hrs,4°C). 40 mls of the dialysed concentrate was combined with approximately 2 ml of the IgG-Sepharose and the mixture incubated end-over-end for 2 hrs(RT). The matrix was exhaustively washed and the recombinant protein eluted using 4M

$MgCl_2$,pH 8.3. The presence of recombinant antigen was confirmed as outlined above.

B. THE PURIFICATION OF THE HUMAN HIV SPECIFIC ANTIBODIES.

The purification of HIV specific human antibodies involved two steps. These are outlined below.

i The preparation of the HIV antigen column.

ii The purification of the HIV specific human antibodies.

1. Preparation of the HIV antigen-Sepharose column.

7.5 mls of the eluted protein was mixed with 2 gms swollen, pre-washed and appropriately equilibrated CnBr-Sepharose (pH8.3). The mixture was mixed end-over-end (2hrs,RT). Unreactive sites were blocked using 0.2M glycine (16hrs, 4°C) and the matrix exhaustively washed as outlined for the IgG-Sepharose column.

2. Purification of HIV specific human antibody.

4 mls of pooled human HIV serum heat treated as outlined above was passed through a PD-10 column equilibrated with freshly prepared 0.05M Phosphate buffer pH7.2 + 0.5M NaCl. The first 3 ml fraction (void volume) was discarded and the next 7.5 mls was collected. 10 mls of the gp120-Sepharose matrix and 7.5 mls of the equilibrated serum were mixed end-over-end for 2hrs at RT. Following extensive washing HIV specific Ig's were desorbed using buffer containing 4M $MgCl_2$ pH8.3. Approximately 2 mg of HIV specific Ig was obtained using this method.

C. THE PRODUCTION OF MOUSE MONOCLONAL ANTIBODY TO THE HUMAN AB1.

The production of Mouse monoclonal antibodies firstly involves the induction of antibodies either by in vivo methods or by in vitro methods.

By way of illustration the in vitro method is described.

Two groups of Balb/c mice were used in this experiment. The first group consisted of mice which had been tolerized to human IgG1. This had been achieved by injecting mice intraperitoneally, 7 days previously, with 10 milligrams of human IgG1. The second group consisted of untolerized mice.

Mouse Ab2 antibodies were induced in the following way. 1.3×10^8 mouse spleen cells were recovered and washed in the incubation medium (Iscoves DMEM medium containing 20% foetal calf serum (FCS), 40% thymus conditioned medium (TCM), 5×10^{-4} 2-mercaptoethanol, 4mML-glutamine 50 IU penicillin and 50 IU streptomycin). HIV specific human immunoglobulins at a concentration of 10 micrograms/ml incubation medium was added to the mouse spleen cells. The total volume used in the incubation of the spleen cells with human antibody varied between 10 and 15 mls. In this example the incubation was allowed to proceed for 7 days in a heated (37°C) CO_2 incubator.

Following incubation the cells were recovered for fusion to either SP2, NS1 or X63-Ag*.653 mouse myeloma

cells. The viability of the spleen cells was found to vary between 70 and 99% and the viability of the myeloma was generally 99%. For the sake of illustration SP2 mouse spleen cells were used though other cells such as rat or human myeloma cells could be used in this procedure. Spleen cells were fused to the myeloma cells using polyethylene glycol 1500/4000 (Boehringer/Mannheim) using standard procedures and following 24 hrs incubation in a CO_2 incubator at 37°C the hybrids were plated out in the incubation medium now containing HAT.

D. RECOVERY OF ANTI-HIV ANTIBODY CLONES PRODUCED IN SITU AS A RESULT OF NATURAL INFECTION.

In addition to the serum HIV antibodies purified by the abovementioned methods it is possible to obtain the human Abl by Epstein-Barr virus (EBV) transformation of human B cells obtained from individuals exposed to the AIDS virus.

By way of illustration the following method was used.

Human peripheral blood lymphocytes (PBL's) were diluted 1:1 in phosphate buffered saline and the red cells removed by centrifugation through a Ficoll-hypaque cushion (Pharmacia).

The PBL's either depleted or not depleted of monocytes and lymphocytes using methods familiar to those skilled in the art, were then transformed using for example the EBV isolate B95-8 in sterile tissue culture media (RPMI-1640 + 5% FCS). In a simple example the B95-8

isolate is made available as a supernate which is mixed with the monocyte/T cell depleted fraction enriched for the B lymphocytes. The cells are grown in this mixture, fed as required, and expanded in 96-well flat bottomed plates prior to fusion with the mouse myeloma cell line such as X63-Ag*.653. Screening is by a commercially available HIV antibody ELISA. Cloning and feeding (Medium containing HAT/HT) is by the usual methods except that non transformed will be selected out by feeding with 1 micromolar Oubain.

All these methods must be carried out in hybridoma facilities suitable for work involving HIV as virus may be shed under these conditions.

E. PRODUCTION OF HUMAN AB1 USING IN VITRO IMMUNIZATION OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES AND/OR SPLENIC LYMPHOCYTES.

HIV specific human Ab1 may also be obtained by in vitro immunization using whole virus or native, recombinant HIV antigens and antigens bound to nitrocellulose. According to one method 3-4 x 10^4 human PBL's or human splenic lymphocytes depleted of monocytes/T lymphocytres using L-Leucine methyl ester can be immunized with small amounts (1 nanogram - 10 micrograms) of HIV antigen. The human Ab1 are monoclonal when the techniques of hybridoma technology as outlined in D. are used. Human Ab1 obtained in this way may be used as the immunogen to produce the Ab2 by either in vivo or in vitro

culture techniques using human cells or cells of other species as the human Abl would house the prototypic paratopes as defined by the foregoing.

Such variations as will be apparent to those skilled in the art from the teaching hereof are deemed to be within the scope of the invention herein disclosed.

CLAIMS:-

1. A method of manufacture of an anti-paratopic antibody comprising the steps of:

(1) selecting from a pool of antibodies occurring in a first species of vertebrate a prototypic set the members of which are antibodies effective in binding a specific antigen (or antigen epitope), and;

(2) utilizing one or more members of said prototypic set, or one or more paratopic fragments thereof, as an immunogen in a host of a different species from the first, or in an in vitro incubation system comprising cells derived from the same or a different species, to produce one or more antibodies having a characteristic which is anti-paratopic with respect to a specific antigen or antigen epitope.

2. A method according to claim 1 further comprising the step of utilizing the anti-paratopic prototypic antibody as an immunogen in a vertebrate of the first species.

3. A method according to claim 1 or claim 2 wherein the prototypic antibodies are human antibodies.

4. A method according to claim 3 wherein the prototypic human antibodies are naturally occurring antibodies.

5. A method according to any one of the preceding claims wherein the antibodies are immunoglobulins.

6. A method according to any one of the preceeding claims wherein the antibodies are human antibodies to HIV.

7. A method of immunising a subject comprising the administration of an anti-paratopic antibody produced according to anyone of claims 1 to 6 to said subject, wherein the prototypic antibody is produced by the species of which the subject is a member.

8. Anti-paratopic antibodies produced according to the method defined by any one of claims 1 to 6.

9. A method of manufacture of an anti-paratopic antibody effective against mammalian infections comprising the steps of:

(1) selecting from a pool of antibodies from infected mammals a prototypic set the members of which are effective in binding the aetiological agent or differentiating epitope, and

(2) utilizing one or more members of said prototypic set, or paratopic fragments thereof, as an immunogen in a host of a different species from the first, or in an in vitro incubation system comprising cells derived from the same or different species, to produce one or more antibodies having a characteristic which is antiparatopic with respect to the said aetiological agent or differentiating epitope.

10. A method of manufacture of an anti-paratopic antibody effective against HIV comprising the steps of:

(1) selecting from a pool of human anti-HIV antibodies, a protoypic set, the members of which are

effective in binding HIV, and

(2) utilizing one or more members of said prototypic set, or paratopic fragments thereof, as an immunogen in a host of a different species or in an in vitro incubation system comprising cells derived from human or a different species to produce antibodies having a characteristic which is anti-paratopic with respect to said immunogen to produce a synthetic replicate of the antigen.

11. A method according to claim 10 wherein the anti-HIV antibodies are enzymatically cleaved to remove the Fc component, and conjugated to immunogenic carriers before step (2).

12. A method according to claim 10 wherein after step (2), the spleen cells of the species employed in step (2) are harvested, fused to myeloma cells and the resulting hybridoma cells are cultured.

13. Monoclonal antibodies produced by the hybridoma cells defined by claim 12.

14. Purified human HIV specific antibodies.

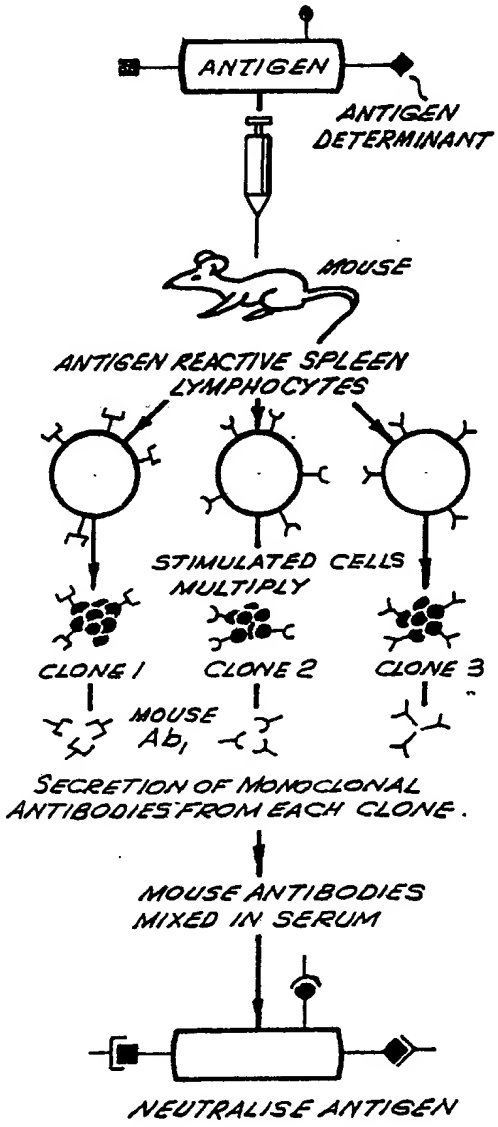
ANTIGEN
ANTIGEN DETERMINANT
MOUSE
ANTIGEN REACTIVE SPLEEN LYMPHOCYTES
STIMULATED CELLS MULTIPLY
CLONE 1
CLONE 2
CLONE 3
MOUSE Ab1
SECRETION OF MONOCLONAL ANTIBODIES FROM EACH CLONE.
MOUSE ANTIBODIES MIXED IN SERUM
NEUTRALISE ANTIGEN

FIG. 1

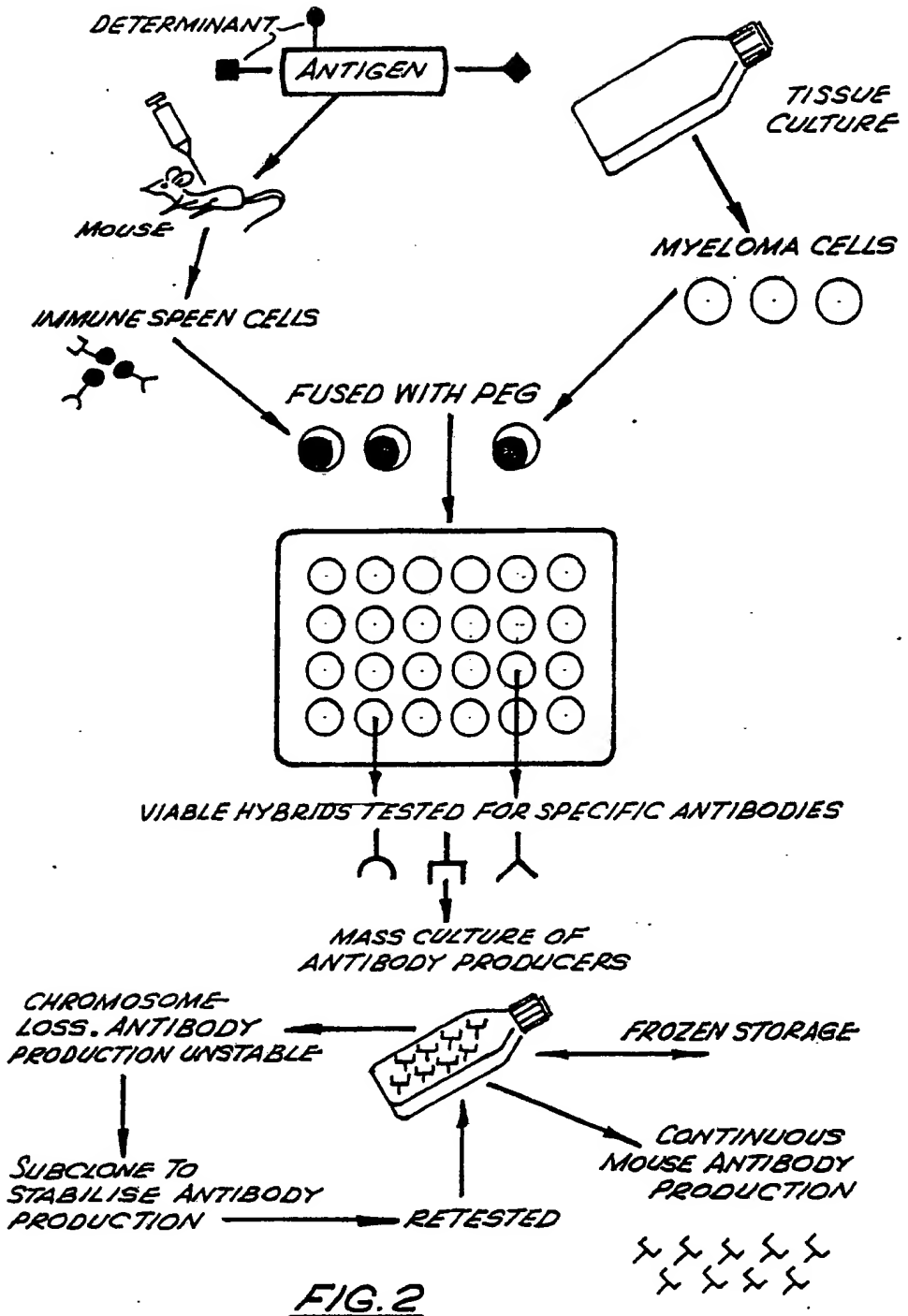
DETERMINANT
ANTIGEN
TISSUE CULTURE
MOUSE
MYELOMA CELLS
IMMUNE SPEEN CELLS
FUSED WITH PEG
VIABLE HYBRIDS TESTED FOR SPECIFIC ANTIBODIES
MASS CULTURE OF ANTIBODY PRODUCERS
CHROMOSOME-LOSS. ANTIBODY PRODUCTION UNSTABLE
FROZEN STORAGE
CONTINUOUS MOUSE ANTIBODY PRODUCTION
SUBCLONE TO STABILISE ANTIBODY PRODUCTION
RETESTED

FIG. 2

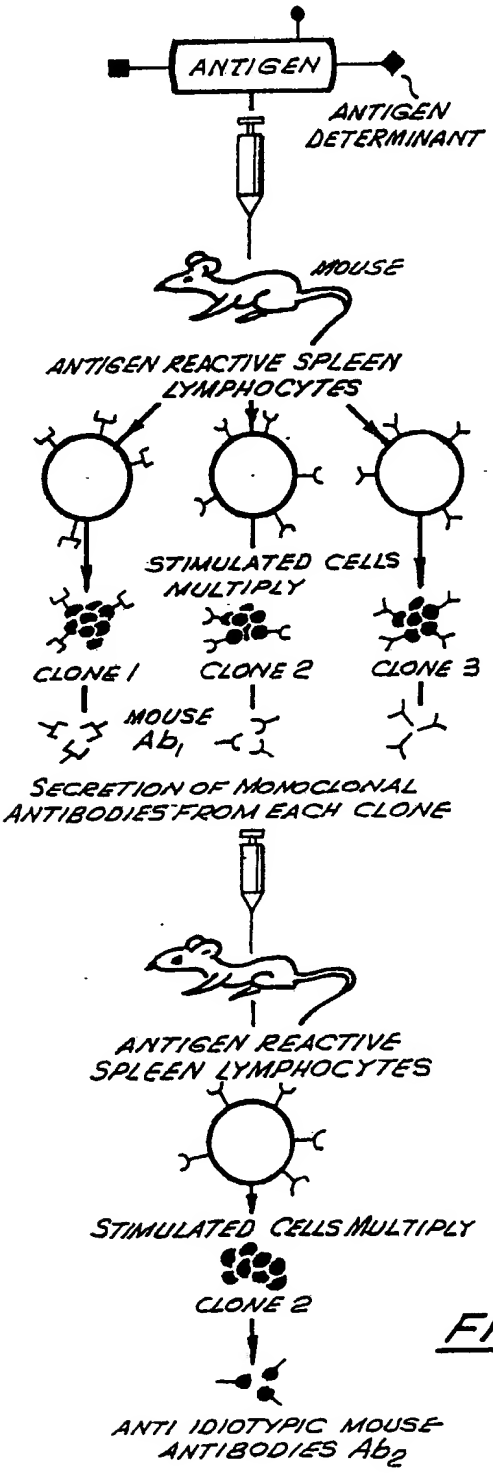
ANTIGEN
ANTIGEN DETERMINANT
MOUSE
ANTIGEN REACTIVE SPLEEN LYMPHOCYTES
STIMULATED CELLS MULTIPLY
CLONE 1
CLONE 2
CLONE 3
MOUSE Ab1
SECRETION OF MONOCLONAL ANTIBODIES FROM EACH CLONE
ANTIGEN REACTIVE SPLEEN LYMPHOCYTES
STIMULATED CELLS MULTIPLY
CLONE 2
ANTI IDIOTYPIC MOUSE ANTIBODIES Ab2

FIG. 3

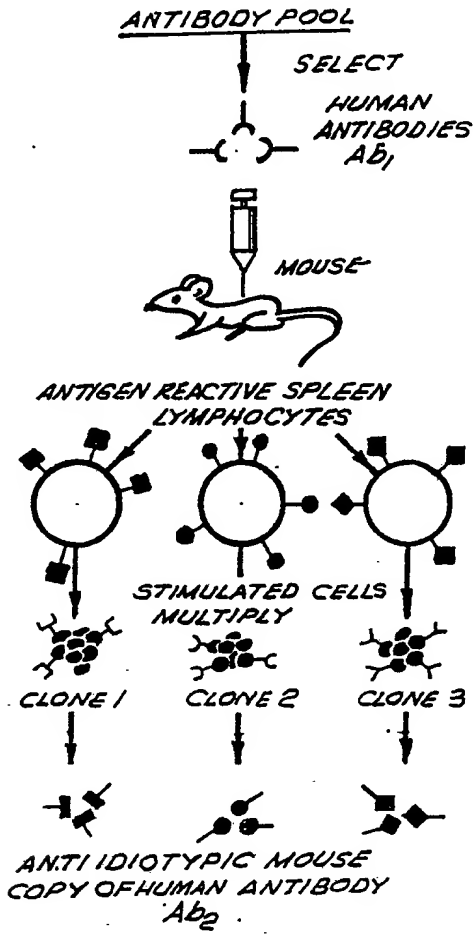
ANTIBODY POOL
SELECT
HUMAN ANTIBODIES
Ab_1
MOUSE
ANTIGEN REACTIVE SPLEEN LYMPHOCYTES
STIMULATED CELLS MULTIPLY
CLONE 1
CLONE 2
CLONE 3
ANTI IDIOTYPIC MOUSE COPY OF HUMAN ANTIBODY
Ab_2

FIG.4

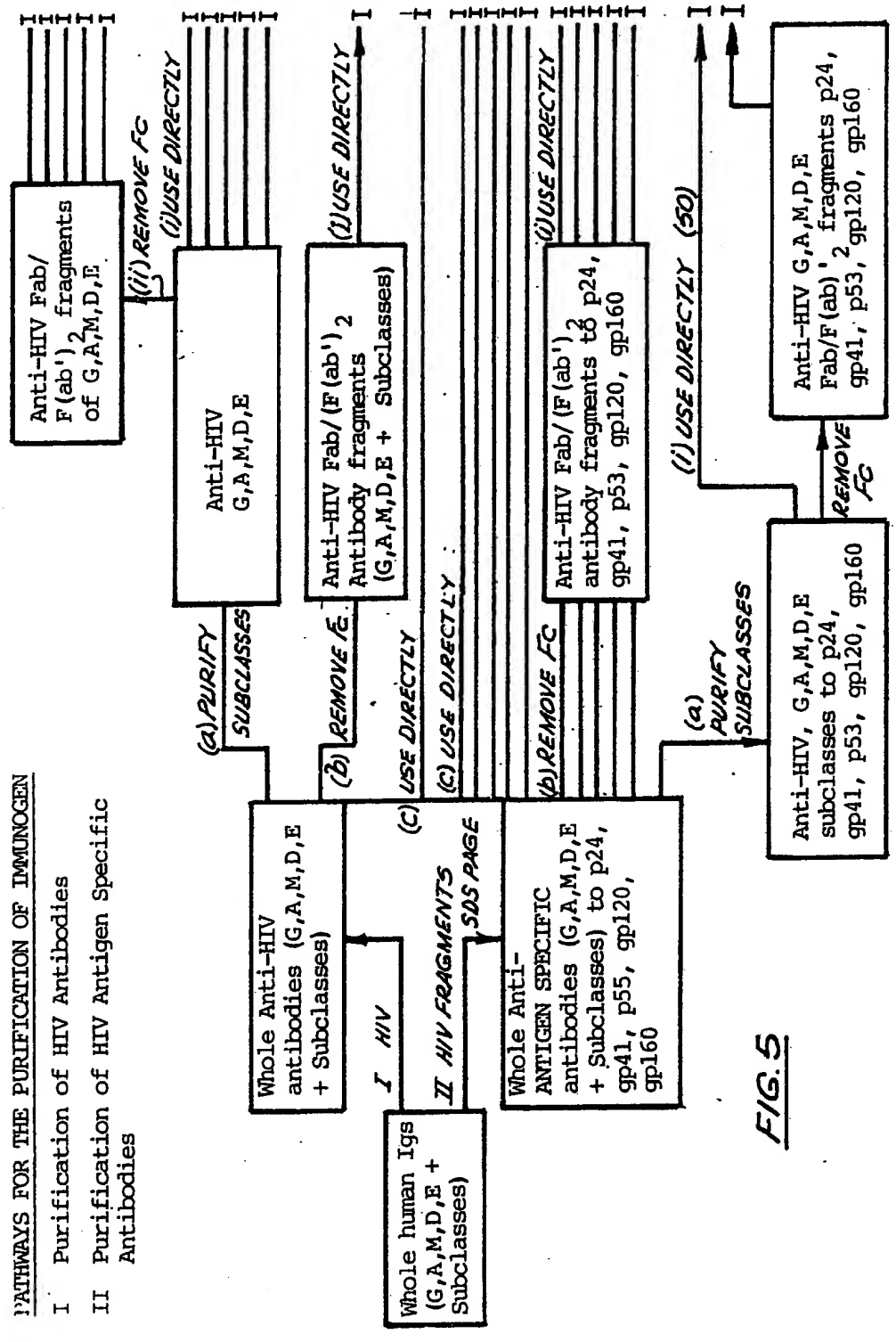
PATHWAYS FOR THE PURIFICATION OF IMMUNOGEN
I Purification of HIV Antibodies
II Purification of HIV Antigen Specific Antibodies
Anti-HIV Fab/ F(ab')2 fragments of G,A,M,D,E
(ii) REMOVE FC
(i) USE DIRECTLY
Anti-HIV G,A,M,D,E
(a) PURIFY SUBCLASSES
Whole Anti-HIV antibodies (G,A,M,D,E + Subclasses)
(b) REMOVE FC
Anti-HIV Fab/ (F(ab')2 Antibody fragments (G,A,M,D,E + Subclasses)
(i) USE DIRECTLY
(c) USE DIRECTLY
I HIV
II HIV FRAGMENTS
SDS PAGE
Whole human Igs (G,A,M,D,E + Subclasses)
(b) REMOVE FC
Anti-HIV Fab/ (F(ab')2 antibody fragments to p24, gp41, p53, gp120, gp160
(i) USE DIRECTLY
Whole Anti-ANTIGEN SPECIFIC antibodies (G,A,M,D,E + Subclasses) to p24, gp41, p55, gp120, gp160
(a) PURIFY SUBCLASSES
Anti-HIV, G,A,M,D,E subclasses to p24, gp41, p53, gp120, gp160
(i) USE DIRECTLY (SO)
REMOVE FC
Anti-HIV G,A,M,D,E Fab/F(ab')2 fragments p24, gp41, p53, gp120, gp160

FIG. 5

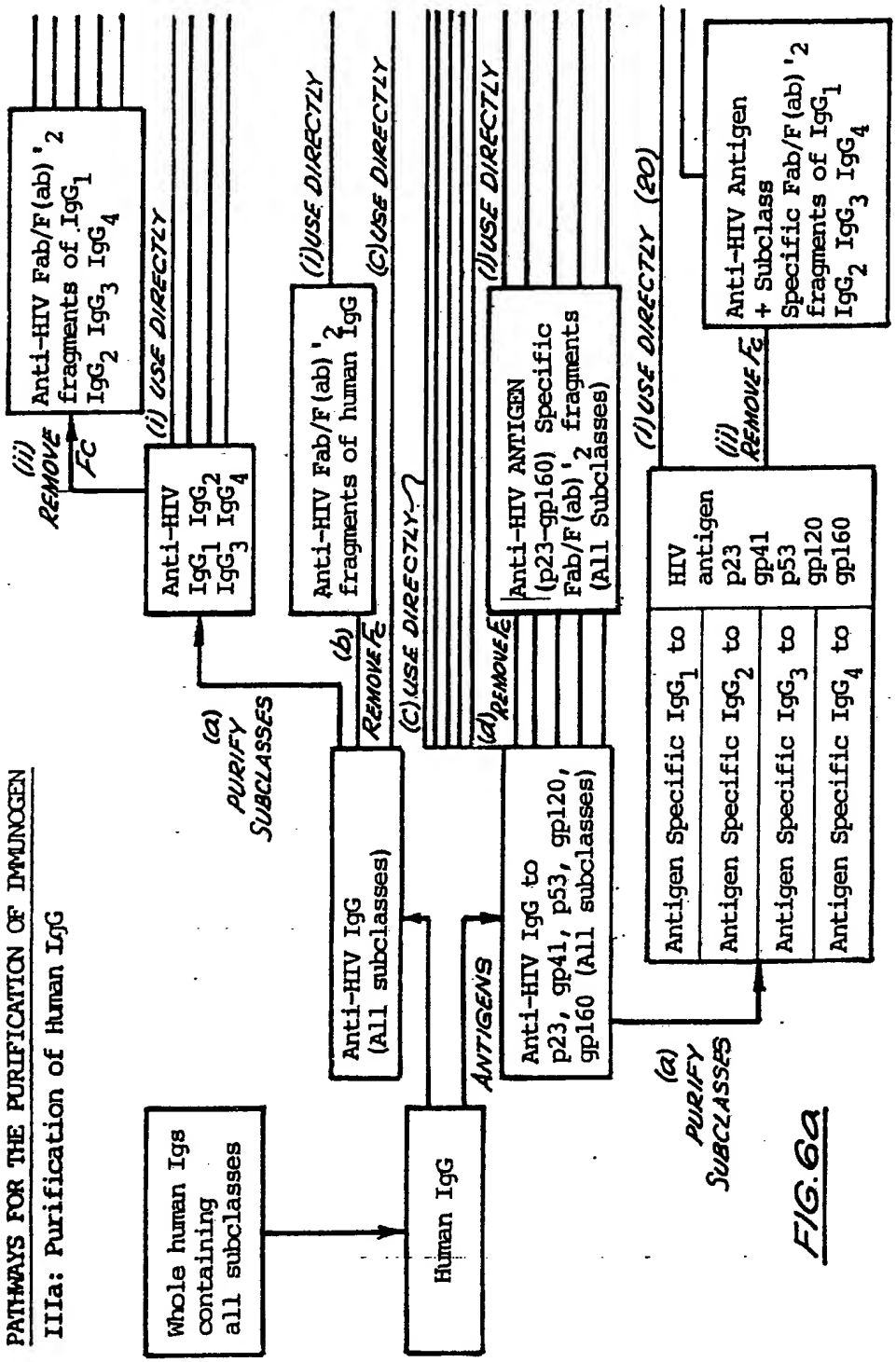
PATHWAYS FOR THE PURIFICATION OF IMMUNOGEN
IIIa: Purification of Human IgG
Whole human Igs containing all subclasses
Human IgG
(a) PURIFY SUBCLASSES
Anti-HIV IgG (All subclasses)
(b) REMOVE Fc
Anti-HIV Fab/F(ab)'2 fragments of human IgG
(c) USE DIRECTLY
(i) USE DIRECTLY
(ii) REMOVE Fc
Anti-HIV Fab/F(ab)'2 fragments of IgG1 IgG2 IgG3 IgG4
ANTIGENS
Anti-HIV IgG to p23, gp41, p53, gp120, gp160 (All subclasses)
(d) REMOVE Fc
Anti-HIV ANTIGEN (p23-gp160) Specific Fab/F(ab)'2 fragments (All Subclasses)
(i) USE DIRECTLY
(ii) REMOVE Fc
Anti-HIV Antigen + Subclass Specific Fab/F(ab)'2 fragments of IgG1 IgG2 IgG3 IgG4
(a) PURIFY SUBCLASSES
Antigen Specific IgG1 to
Antigen Specific IgG2 to
Antigen Specific IgG3 to
Antigen Specific IgG4 to
HIV antigen p23 gp41 p53 gp120 gp160

FIG. 6a

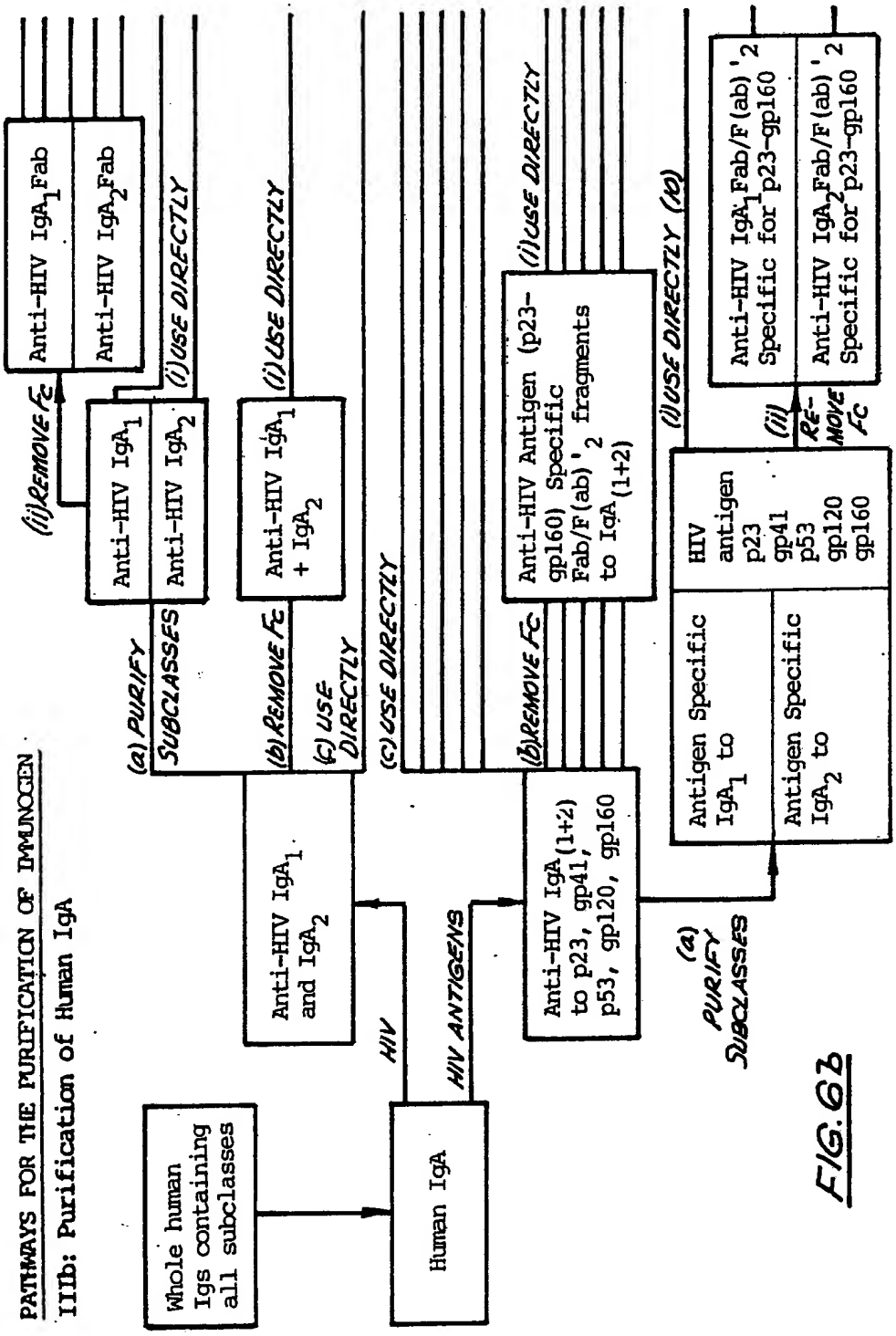
PATHWAYS FOR THE PURIFICATION OF IMMUNOGEN
IIIb: Purification of Human IgA
Whole human Igs containing all subclasses
Human IgA
HIV
HIV ANTIGENS
(a) PURIFY SUBCLASSES
Anti-HIV IgA1 and IgA2
(b) REMOVE Fc
(c) USE DIRECTLY
Anti-HIV IgA1 + IgA2
(i) USE DIRECTLY
(ii) REMOVE Fc
Anti-HIV IgA1
Anti-HIV IgA2
Anti-HIV IgA1 Fab
Anti-HIV IgA2 Fab
(i) USE DIRECTLY
(c) USE DIRECTLY
Anti-HIV IgA (1+2) to p23, gp41, p53, gp120, gp160
(b) REMOVE Fc
Anti-HIV Antigen (p23-gp160) Specific Fab/F(ab)'2 fragments to IgA (1+2)
(i) USE DIRECTLY
(a) PURIFY SUBCLASSES
Antigen Specific IgA1 to
Antigen Specific IgA2 to
HIV antigen p23 gp41 p53 gp120 gp160
(ii) USE DIRECTLY (a)
(iii) REMOVE Fc
Anti-HIV IgA1 Fab/F(ab)'2 Specific for p23-gp160
Anti-HIV IgA2 Fab/F(ab)'2 Specific for p23-gp160

FIG. 6B

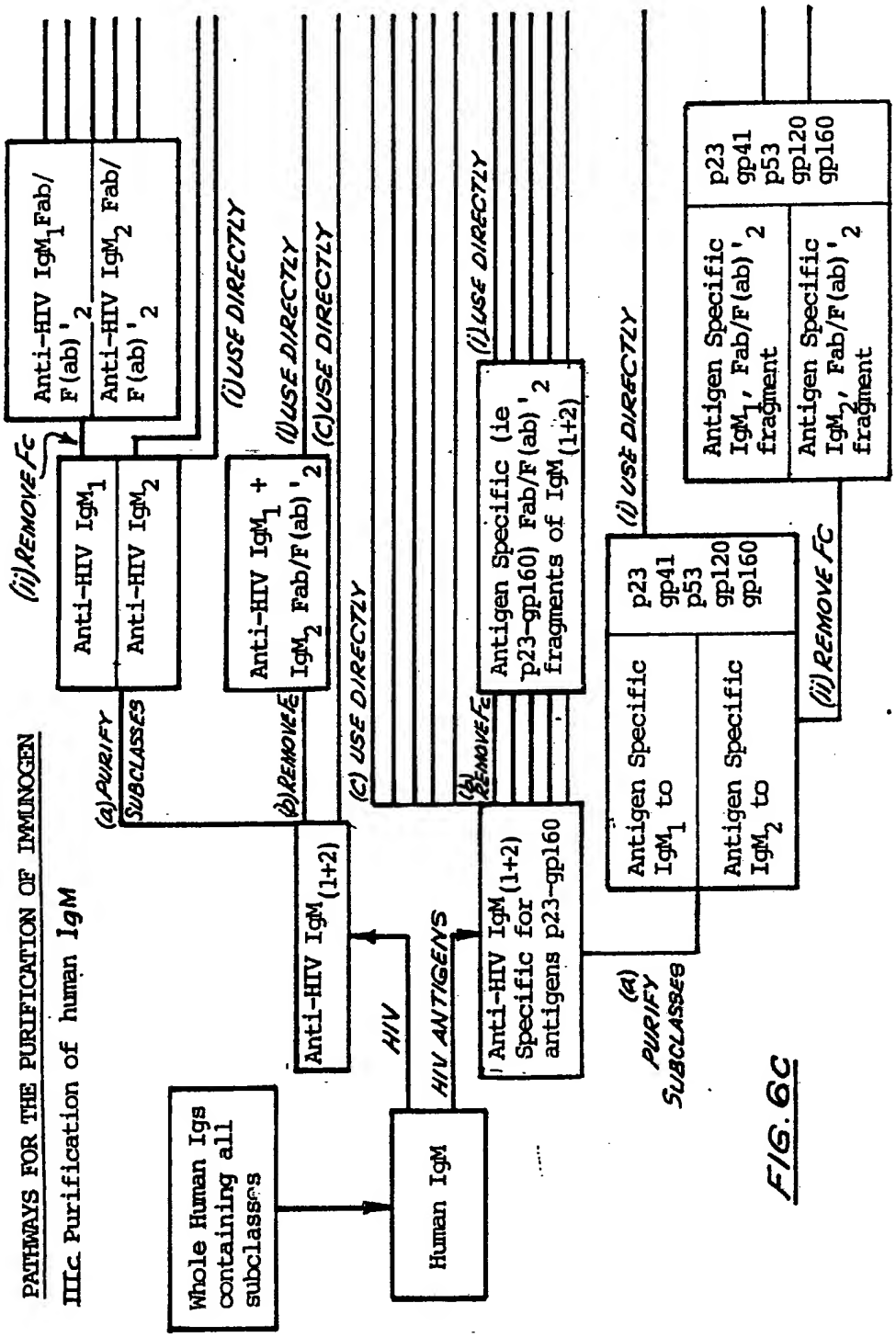
PATHWAYS FOR THE PURIFICATION OF IMMUNOGEN
IIIc Purification of human IgM
Whole Human Igs containing all subclasses
Human IgM
HIV
HIV ANTIGENS
Anti-HIV IgM(1+2)
Anti-HIV Specific for antigens p23-gp160 IgM(1+2)
(a) PURIFY SUBCLASSES
(b) REMOVE Fc
(c) USE DIRECTLY
(i) USE DIRECTLY
(ii) REMOVE Fc
Anti-HIV IgM1
Anti-HIV IgM2
Anti-HIV IgM1 + IgM2 Fab/F(ab)'2
Anti-HIV IgM1 Fab/F(ab)'2
Anti-HIV IgM2 Fab/F(ab)'2
Antigen Specific (ie p23-gp160) Fab/F(ab)'2 fragments of IgM(1+2)
Antigen Specific IgM1 to
Antigen Specific IgM2 to
p23 gp41 p53 gp120 gp160
Antigen Specific IgM1, Fab/F(ab)'2 fragment
Antigen Specific IgM2, Fab/F(ab)'2 fragment

FIG. 6C

INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 88/00074

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] C07K 15/12, C12P 21/00, C12N 15/00, 5/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	WPI, WPIL, USPA : Keywords: HIV, Human Immunodeficiency Virus, HTLV-III, Human T-Cell Lymphotropic Virus Type-III, LAV Lymphadenopathy Associated Virus, ARV, AIDS-related Virus, AIDS, Acquired Immune Deficiency Syndrome and Antibody

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

AU : C07K 15/12, 7/00, G01N 33/54, 33/569, 33/571, 33/577
Chemical Abstracts : Keywords : as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
P,X	Chanh, T.C. et al. "Monoclonal anti-idiotypic antibody mimics the CD4 receptor and binds human immunodeficiency virus" Proc. Natl. Acad. Sci. USA, Volume 84, issued June 1987, (National Academy of Sciences, USA) see pages 3891 to 3895.	(1-13)
P,X	del Guergo, P. and M. Zonetti. "The CD4 molecule, the human immunodeficiency virus and anti-idiotypic antibodies". Immunology Today, Volume 8, No.8, issued 1987, (Elsevier Publications, Cambridge) see pages 204 to 205.	(1-13)
P,X	Zhou, E. et al. "Immune Response to Human Immunodeficiency Virus : In Vivo Administration of Anti-Idiotype Induces an Anti-gp160 Response Specific for a Synthetic Peptide" Journal of Immunology, Volume 139, issued 1 November 1987, (Williams & Wilkins Co. USA), see pages 2950 to 2956.	(1-13)
P,X P,Y	Derwent Abstract Accession No. 87-271905/39, Class B04, DE,A, 3609271 (BOEHRINGER MANNHEIM GMBH) 24 September 1987 (24.09.87)	(1-5,8,9) (6,10,11-13)
X	AU,A, 36143/84 (THE WISTAR INSTITUTE) 23 May 1985 (23.05.85) (continued)	(1-13)

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
5 July 1988 (05.07.88)	(19.07.88) 19 JULY 1988
International Searching Authority	**Signature of Authorized Officer**
Australian Patent Office	JOHN ASHMAN

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X Y	AU,A, 37844/85 (SCRIPPS CLINIC AND RESEARCH FOUNDATION) 4 July 1985 (04.07.85)	(1-3,5,8,9) (4,6,10-13)
X Y	AU,B, 21712/83 (566246) (YESHIVA UNIVERSITY) 7 June 1984 (07.06.84)	(1-5,8,9) (6,10-13)
X Y	AU,A, 47110/85 (IMMUNOMEDICS INC.) 10 April 1986 (10.04.86)	(1-5,8,9) (6,10-13)
X Y	AU,A, 47773/85 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK) 13 March 1986 (13.03.86)	(1-5,8,9) (6,10-13)

(continued)

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 7, because they relate to subject matter not required to be searched by this Authority, namely:

it involves a method of treatment of the human or animal body by therapy.

2. [ ] Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers ....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X Y	Hay, F.C. et al. "Idiotype Vaccines" in Immune Intervention, published in 1984, by Academic Press (London) see pages 117 to 138.	(1-5,8,9) (6,10-13)
X Y	McDougall, J.S. et al. "Binding of the Human HTLV-111/LAV/ARV/HIV to the CD4 (T4) Molecule : Conformational Dependence, Epitope Mapping, Antibody Inhibition, and Potential for Idiotypic Mimicry" Journal of Immunology, Volume 137, No.9, issued 1 November 1987 (Williams & Wilkins Co., USA) see pages 2937 to 2944.	(1,3,4,6,8-10) (11-13)
X Y	Kennedy, R.C. et al. "Immune Response to Hepatitis B Surface Antigen : Enhancement by Prior Injection of Antibodies to the Idiotype" Science, Volume 221, issued 26 August 1983, (American Association for the Advancement of Science, USA) see pages 853 to 855 .	(1-5,8,9) (6,10-13)
X Y	Dreesman, G.R. and R.C. Kennedy. "Anti-Idiotypic Antibodies : Implications of Internal Image-Based Vaccines for Infectious Diseases" The Journal of Infectious Diseases, Volume 151, No.5, issued May 1985 (University of Chicago Press, USA) see pages 761 to 765.	(1-5,8,9) (6,10-13)
X Y	AU,A, 53200/86 (INSTITUT PASTEUR AND CENTRE NATIONAL DE LA RECHERCHE SCIENTIFQUE) 31 July 1986 (31.07.86)	(14) (6,10-13)
X Y	AU,A, 56126/86 (INSTITUT PASTEUR AND CENTRE NATIONAL DE LA RECHERCHE SCIENTIFQUE) 23 October 1986 (23.10.86)	(14) (6,10-13)
X Y	AU,A, 56363/86 (F. HOFFMANN-LA ROCHE AND CO. AKTIENGESELLSCHAFT AND THE GOVERNMENT OF THE UNITED STATES AS REPRESENTED BY THE SECRETARY OF THE DEPARTMENT OF HEALTH AND HUMAN SERVICES) 23 October 1986 (23.10.86)	(14) (6,10-13)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/AU 88/00074

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members		
AU 36143/84	DK 3102/85	EP 141783	FI 852683
	HU 38841	IL 73428	PT 79455
	WO 8502121	ZA 8408713	PT 79455
	US 4731237		
AU 37844/85	DK 3896/85	EP 167596	WO 8502909
	ZA 8410147		
AU 21712/83	CA 1215009	DK 5573/83	EP 113431
	ES 527730	FI 834424	IL 70366
	JP 59122951	NO 834434	NZ 206467
	US 4690905	US 4692416	
AU 47110/85	EP 176355	IL 76459	JP 61084561
	US 4699880	ZA 8507321	
AU 47773/85	EP 191846	WO 8601539	US 4614260
	FR 2554192	GB 2149030	JP 60172737
	SE 8405325		
AU 53200/86	DK 4509/86	EP 211022	ES 557357
	WO 8604336		
AU 56126/86	DK 1699/86	JP 61286756	PT 82396
	ZA 8602812		
AU 56363/86	DK 1772/86	EP 199301	ES 554155
	ES 8706209	FI 861626	JP 62012799
	NO 861546		

END OF ANNEX